The University of Chicago

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A DISSERTATION

SUBMITTED TO THE FACULTY
OF THE OGDEN GRADUATE SCHOOL OF SCIENCE
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF PATHOLOGY

BY
HARRY LEE HUBER

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THE PHARMACOLOGY AND TOXICOLOGY OF COPPER SALTS OF AMINO ACIDS

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS XVI

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Since the middle of the nineteenth century certain events have stimulated at irregular intervals the investigative work on copper, until now a remarkably voluminous and contradictory literature has developed. At first the chief interest in copper was its reputed harmfulness to the health of man when used in the greening of certain vegetables for market, and this practice led, after many years of investigation by the French government to the passage of a law prohibiting the use of copper in canned vegetables. Later, however, this law was repealed as the evidence for the harmful influence of copper when taken in such small doses was inconclusive, but in 1911 in the United States, Taylor, Long and Chittenden reported some work to show that "even such small quantities of copper (10 to 12 mgm.) may have a deleterious action and must be considered injurious to health."

In France again the work on copper received another impetus in the early 80's when it was found by Millardet to be an effective agent for combating certain plant growths which attacked vineyards. After this discovery various preparations of copper were used as germicidal agents and after the work of Naegeli on Spirogyra, copper came into common use as an effective agent in combating vineyard diseases and in the purification of city water supplies. The more recent contradictory works of Moore,

and of Clark and Gage show that there is still a question as to the value of copper in the purification of water supplies.

Although copper has been recommended for many years as a therapeutic agent in such diseases as typhoid (Burg, Walker, Wilcke), cholera (Cummins, Burg), cancer (Loeb) (Manara), diarrhea and dysentery (Cummins, Gresswell), leprosy (Takano), actinomycosis, sporotrichosis (Bevan), etc., it is only in recent years that an attempt has been made to study systematically the therapeutics of various copper compounds. In 1911, Corper began in this laboratory a series of experimental studies on the chemotherapy of tuberculosis, using some copper salts of amino-acids, but his work failed to show any specific curative effects with the copper salts employed. A year or two later von Linden, Strauss, Meissen, Bodmer, and Eggers reported very striking results in the treatment of pulmonary and skin tuberculosis by using a specially prepared copper-lecithin compound. Pohl, Benzi, and others, however, could see no improvement in their tuberculous patients after using this or similar preparations. Recently Kogo, Sato, Omura and other Japanese investigators have reported marked improvement in cases of pulmonary and skin tuberculosis after using a preparation of copper and cyanide which they call cyano-cuprol. This work is still too new to be either accepted or rejected.

Experimental work has shown that the value of copper as a fungicidal, bactericidal, or therapeutic agent depends on bringing the copper in contact with the organism to be destroyed in the proper form and concentration. Certain preparations have been found very effective in destroying fungus growths, and Bordeaux mixture is commonly used for this purpose, but Malverzin and Perrin recommended other copper compounds which are said to be more effective, although Bedford and Pickering, after studying the effects of many fungicides, come to the conclusion that the effectiveness depends entirely on the concentration of copper. There is also a discussion as to the bactericidal properties of different copper salts. Gildersleeve found copper sulphate to be more active, due to its more rapid electrolytic dissociation, copper chloride, copper acetate and copper

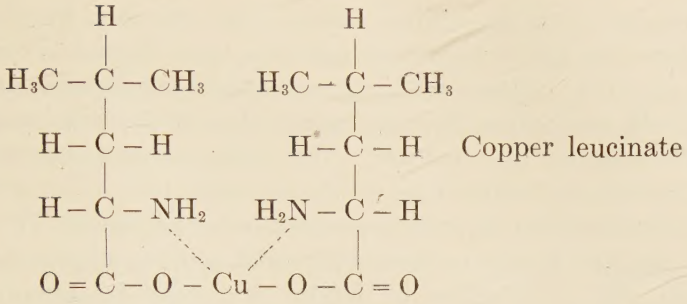
nitrate following in the order given. This finding is in accord with that of Krönig and Paul. Shaw and Mackenzie report that copper-alanine is very lethal in low concentrations to the lower organisms, while DeWitt and Sherman were unable to find much difference in the activity of copper sulphate and copper chloride on certain organisms. A great deal of work has been done on experimental animals to determine the action of different copper compounds but the methods of experimentation are so variable that comparisons are hard to make.

Thus, Taylor found copper-oxide, copper-tartrate and copper-phyllocyanat equally toxic for rabbits, Lehmann found that copper acetate, sulphate, carbonate, citrate, chloride and succinate differed in no way in toxicity. Mock found no difference in the action of copper fatty acids and other copper salts, while Baum and Seeliger found copper oleate most toxic, copper acetate and copper sulphate somewhat less toxic, and copper-haemol almost without action. Brandl found copper oxydnatron most toxic, then copper acetate oleate and stearate in the order given. Tschirch and Lang were unable to see any difference in the different copper compounds. Feltz and Ritter decided that the acetate was more toxic than the sulphate and that the insoluble forms of copper albuminate were without action. Filehne also found that cupratin (made by heating sodium albuminate with copper sulphate) was almost harmless and says, "It seems that foods containing copper combined with albumins are harmless." Filehne also found that copper-sodium-tartrate was more toxic internally and less toxic intravenously than copper-potassium-tartrate. Burq and Ducom found on effect from feeding metallic copper while De Moor found it toxic. Trolldenier noted that copper oleate produced greater pathological changes than other copper salts. Uhl prepared a copper peptone compound which he found to be almost non-toxic. Long and Taylor reported that there was a distinct difference in the action of copper when given as the sulphate and as the compound found in greened vegetables. The different results obtained by these various observers are hard to explain, for it seems that copper, like mercury and other heavy metals, must form compounds which vary in toxicity.

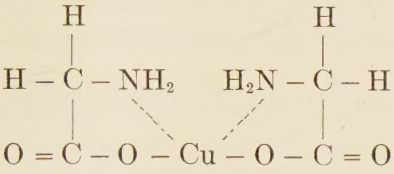
In order to throw more light on some of these questions and to compare the toxic properties of copper sulphate with some of the copper compounds of pure amino-acids, (glycine, glutaminic acid, and leucine), the following experimental work was undertaken. Amino acid combinations with copper were chosen for several reasons: No similar experimental work has thus far been reported, to our knowledge, in which copper compounds of pure amino acids have been used; the amino-acid radicals are utilizable by the organism and their use eliminates the introduction of radicals which may modify the reaction; and the therapeutic value of copper, as indicated by the reports of recent investigators, may be enhanced by its introduction in combination with radicals which are non-toxic and are easily utilizable. It was thought that possibly amino acid salts of copper might be more readily absorbed, or penetrate the cells better, or be less toxic than other salts, since the acid radical is one constantly present in the blood and tissues. To insure uniformity practically all the work was done on guinea pigs. This work naturally falls into two parts, the investigation of the acute toxic properties and of the chronic toxic properties of these compounds.

PREPARATION OF THE COPPER COMPOUNDS

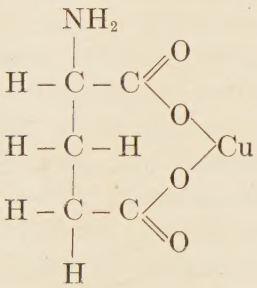
Four copper salts including one inorganic preparation, copper sulphate, and three organic preparations, copper glycinate, copper glutaminate, and copper leucinate, were used in the experimental work. The organic preparations were chosen because they represent an easily soluble and a difficulty soluble double amino-acid copper complexes, copper leucinate and glycinate, and a single amino-acid copper complex, copper glutaminate. The formulae for these compounds are:



Copper leucinate



Copper glycinate



Copper glutaminate.

These organic preparations of copper were prepared in this laboratory. The leucine was prepared by hydrolysis of proteins by Dr. Koch of the physiological chemistry department. The glycine was prepared synthetically by Dr. Witzemann of the Sprague Institute and the glutamic acid prepared by myself from wheat glutin. As the glutamic acid is isolated in the form of glutaminic hydrochloride, it is necessary to remove the chlorine before making the copper salt. The chlorine was removed with an excess of freshly prepared Ag_2O and the excess Ag precipitated with H_2S. From this point the preparation was the same as with the glycine and the leucine. These amino acids were dissolved in a large excess of water and were brought to a boiling temperature. To this was added freshly prepared,

sulphate-free CuO in boiling water. The mixture was boiled and stirred for fifteen to twenty minutes, then filtered to remove excess of CuO. These solutions were then evaporated on the steam bath until crystallization began, then were cooled and the crystals collected by filtration. The mother liquor was evaporated further and other crops of crystals collected. The crystals were then dried and kept in a dessicator until used. The leucinate and the glycinate form blue salts and the glutaminate a greenish salt. According to Kober and Sugiura the mono-basic copper-glycinate is soluble, the mono-basic copper-leucinate very insoluble and the dibasic copper-glutaminate soluble.

METHOD OF MAKING COPPER DETERMINATIONS

The method of making copper determinations was that used by Corper, with slight modifications. The organic material was destroyed completely, as described by Corper, in Kjeldahl flasks with nitric and sulphuric acids, and the sulphuric acid evaporated to a small volume by forcing air down the neck of the flask. This mixture was then washed into a 200 cc. wide-mouthed bottle with copper-free redistilled water, and neutralized to litmus with copper-free 50 per cent sodium hydroxide. After neutralization, one cc. of sulphuric acid (concentrated sulphuric acid diluted with an equal volume of water) and sufficient distilled water were added to bring the volume to about 80 cc. The solution was then heated to about 70°C. and the copper separated from the heated solution by electrolysis. A piece of platinum foil about 2 cm. square suspended by a platinum wire through a small hole in the side of the bottle was used as a depositing electrode. The other electrode was a platinum disc 1.5 cm. in diameter, so arranged that it could be rotated several hundred times a minute. This rotation insured complete mixing of the electrolized solution, and, with a storage battery current of 2 to 2.5 volts and 0.2 amperage, the small amounts of copper were completely deposited in two to three hours, as shown by controls.

After the deposition was complete the method as used by Corper was followed. In all of this work care was taken to

use only copper-free reagents so that quantitative accuracy to about 0.03 mgm. of copper was possible. Recently, Teruuchi and Omura have devised a new method of making copper determinations, which they claim is quantitatively accurate to 0.01 to 0.003 mgm. of copper.

THE EFFECT OF COPPER ON THE EYE OF RABBITS

The minimal irritating concentration of copper sulphate, copper-glutaminate, copper glycinate, and copper leucinate on the eye of rabbits was determined by dropping 1 cc. of distilled water solutions of these salts, made up with different definite concentrations of copper, into the conjunctiva. The amount and rapidity of inflammation and the effect on the lacrimal gland served as an index of the degree of irritability. There was a slight variation in different rabbits and in the same rabbit at different times but, taken as a whole, the results as given in table 1 indicate that the degree of irritability depends on the concentration of copper and not on the salt used, as the same concentration of copper in all the different salts, produced effects which were not distinguishable. It will be noted that the higher concentrations of copper-leucinate and of copper glutaminate are not used, as these salts are not easily soluble in distilled water. All of the higher concentrations produced lacrimation within a few seconds.

TABLE 1

The effect of copper on the eye of rabbits

	CONCENTRATION OF Cu			
	1 per cent	0.1 per cent	0.05 per cent	0.01 per cent
Cu-sulphate	Hyperemia in 2 minutes	Slight hyperemia in 5 minutes	Slight hyperemia 8 minutes	No effect
Cu-glutaminate			Slight hyperemie 8 minutes	No effect
Cu-glycinate	Hyperemia in 2 minutes	Slight hyperemia in 5 minutes	Slight hyperemia 8 minutes	No effect
Cu-leucinate				No effect

THE LOCAL TOXIC ACTION OF COPPER

In 1914 Corper reported the use of the intracutaneous injection method to determine differences in the local toxic action of chemicals. This method was used in studying the local toxic action of copper. These intracutaneous inoculations of 0.2 cc. were made with a 1 cc. tuberculin syringe, in the backs of white guinea pigs, varying concentrations (1., 0.1, 0.01, 0.001 per cent copper concentration) of a water solution of copper sulphate being used on one side of the back and corresponding concentrations of water solutions or suspensions of the copper-amino-acids on the other side of the back, equal concentrations opposite each other. Before inoculation, the hair was shaved from the back and the thickness of the skin at each point of injection was determined by pinching up the skin in a fold and measuring with a micrometer. After the inoculation, daily observations and measurements were made for five to seven days. Some of the animals were killed within forty-eight hours after inoculation and the inoculated areas preserved in Zenker's fluid for histological examination. The higher concentrations of both the copper sulphate and the copper amino-acid always caused a necrosis of the tissue, and varying degrees of induration at the point of inoculation. The lower concentrations caused no necrosis and little or no induration. The gross comparisons did not show any marked differences in the reaction of the equal copper concentrations of the different copper salts. In a few cases the copper-amino acid solution produced a more hemorrhagic lesion, but this was not constant. The degree and kind of necrosis seemed to be alike with the higher concentrations of all the preparations used. No differences in reaction could be made out histologically, thirty-six hours after the inoculation. With all concentrations of all the preparations there was a slight edema of the lower layers of the corneum and a more or less marked polymorphonuclear infiltration and edema in the corium adjacent to the inoculated area. With the higher concentrations of all the preparations there was evidence of beginning degeneration in the corneum at the site of inoculation and the capillaries in

the corium surrounding the area were more distended than with the lower concentrations. Preparations of inoculated areas, taken seven to eight days after inoculation, showed no changes with the lower concentrations and only scar tissue with the higher concentrations.

So far as the local toxic action of the different copper preparations on the skin is concerned, it seems that there are no distinguishable differences in reaction.

SUBCUTANEOUS INJECTIONS OF COPPER

Filehne, Brandl, Pinkus, Sellei, De Moor, Uhl, Chittenden, Corper and others have determined the toxic dose of many copper preparations but, as most of them fail to state definitely all the conditions of their experiments, it is impossible to interpret their findings. Table 2 shows briefly the results of their experiments.

As these findings seem to show that different copper preparations have different degrees of toxicity, a series of experiments was conducted to determine the toxic dose of copper sulphate, copper leucinate, copper glycinate, and copper glutaminate for guinea pigs. These copper solutions, made up with distilled water and calculated in percentage of copper, were injected into the subcutaneous tissue of the axilla and groin. As the higher concentrations of copper solutions cause a necrosis at the point of injection and as Novy has recently shown that relatively large amounts of pure distilled water may be injected even intravenously without harm, an attempt was made to insure complete absorption of the copper by making higher dilutions than have been used by most of the other workers. This made it necessary to make injections at several points and consequently insured a more rapid absorption. The symptoms following the injections when death occurred within ten to twelve hours were the same for all of the copper preparations used; namely, marked irritation and restlessness for about an hour; then a huddling together, the hair on the neck and back standing erect, until death intervened. A few of the pigs developed marked spasmodic contractions of the hind legs and a few which were

TABLE 2

Determinations of the toxic dose of copper

OBSERVER	DATE	COPPER PREPARATION	ANIMAL	METHOD	MILLIGRAMS OF Cu PER KILO	LIVED
Filehne	1895	Copper K tartrate			65.0	
		Copper Na tartrate			30.0	
		Copper Na tartrate	Rabbit	Intravenous	3.0	1½ hours
		Copper Na tartrate	Rabbit	Subcutaneous	9.0	24 hours
		Copper Na tartrate	Rabbit	Per os	50 0	
Brandl	1896	Copper Na tartrate	Dog	Intravenous	3.0	
		Copper Na tartrate	Dog	Subcutaneous	21.0	4 days
Sellei	1913	$CuCl_2$	Guinea-pig	Subcutaneous	50.0	5 to 8 hours
		$CuSO_4$	Guinea-pig	Subcutaneous	25.0	36 to 48 hours
Chittenden	1913	$CuSO_4$	Rabbit	Intravenous	7.5	35 minutes
		$CuSO_4$	Rabbit	Intravenous	4.1	4 hours
		$CuSO_4$	Dog	Intravenous	1.4	
Corper	1914	Copper amino-acid mixture	Mice	Intraperitoneal Intramuscular	5 0–10 0	
Pinkus	1914	$CuSO_4$	Guinea-pig	Intravenous	1 5	
		$CuSO_4$	Guinea-pig	Subcutaneous	17.0–20.0	
		$CuSO_4$	Guinea-pig	Per os	80	
Uhl	1914	Cu peptone	Rabbit	Subcutaneous	23	No effect
		Cu peptone	Rabbit	Intravenous	20	No effect

given larger doses of copper developed paralyses of both hind legs. This latter observation has been noted by several of the early workers (Filehne, Tschirch), and led them to believe that copper is a neuro-toxin, but as these paralyses develop only occasionally, this conclusion does not seem justified. The autopsy findings in pigs dying within ten hours after the injection show no constant variations for the different copper salts. A few typical protocols will give the usual findings:

Guinea-pig. Weight 500 grams. Injected 8 mgm. Cu as copper glutaminate per kilo in 18 cc. of distilled water. Died three hours after injection. No spasmodic contractions—no paralyses. Autopsy findings: Slight edema at points of injection, no excess fluid in peritoneal or pleural cavity, gastro-intestinal tract hyperemic, petechial hemorrhages in serosa of small intestine, gall bladder distended, urinary bladder empty, kidney, liver, spleen, lungs, and heart show no changes.

Guinea-pig. Weight 520 grams. Injected 8 mgm. Cu as copper glycinate per kilo in 17.5 cc. of distilled water. Died sixteen hours after injection. No spasmodic contractions, no paralyses. Autopsy findings: Marked edema of abdominal wall at site of injections, peritoneal exudate 15 cc. hemorrhagic, gastro-intestinal tract hyperemic, no petechial hemorrhages. Gall bladder empty, urinary bladder 2 cc. of a blood tinged urine, kidneys pale, no changes in other organs.

Guinea-pig. Weight 860 grams. Injected 8 mgm. Cu as copper sulphate per kilo in 28 cc. of distilled water. No spasmodic contractions, no paralyses. Died three and one-half hours after injection. Autopsy findings: Slight edema at points of injection, peritoneal exudate (15 cc.) clear, gastro-intestinal tract hyperemic, gall bladder empty, urinary bladder empty, no petechial hemorrhages, no changes in kidney, liver, spleen, lungs or heart.

Guinea-pig. Weight 430 grams. Injected 8 mgm. Cu as copper leucinate per kilo in 17 cc. of distilled water. No spasmodic contractions, no paralyses. Died three hours after injection. Autopsy findings: Slight edema at points of injection, no peritoneal exudate, hyperemia of gastro-intestinal tract, no petechial hemorrhages, bladder distended, urinary bladder empty, no changes in kidney, liver, spleen, lungs or heart.

The only constant difference in findings with the various salts was the greater edema at the injection point produced by the

copper glycinate. Table 3 shows the effect of varying the dosage, and the dilution in these subcutaneous injections.

Some observers (Brandl, Lehmann, Philippeaux) have noted that smaller doses of copper are more quickly fatal in fasted animals than in well fed animals. In the few animals tried here the reverse was true with subcutaneous injections, the well fed animals, with one exception, dying in half the time required with the fasted animals.

In order to show the effect of dilution on the toxic dose of copper, two pigs were injected with equal volumes of a copper glutaminate solution containing 0.011 per cent of copper, and one was injected with a solution containing 0.033 per cent cop-

TABLE 3

Acute intoxication with copper

DILUTION	Cu	Cu-GLYCINATE	Cu-GLUTAMINATE	Cu-LEUCINATE	Cu-SULPHATE
per cent Cu	*mgm. per kilo*				
0.020	10	Died in 1 hour	Died in 8 hours	Died in 4½ hours	Died in 1 hour
0.020	8	Died in 16 hours	Died in 3 hours	Died in 3 hours	Died in 3 hours
0.020	4	Died in 42 hours	Died in 41 hours	Died in 18 hours	Died in 34 hours
0.020	4	Died in 42 hours	Died in 80 hours		
0.020	4		Died in 90 hours		
0.032	6	Died in 35 hours	Died in 50 hours		
0.032	6		Died in 72 hours		
0.032	6		Died in 26 hours		
0.032	6		Died in 20 hours		

per. The pigs receiving the higher dilution died on the third day, while the pig receiving the lower dilution apparently recovered from the effect of the injection and was killed on the fifth day on account of the large area of necrosis at the places of injection. The autopsy findings in these pigs differed greatly, there being no changes in the viscera of the pig receiving the lower dilution, while in those receiving the higher dilutions the hyperemic intestinal tract and parenchymatous changes in the kidney, spleen and liver, were very evident.

Klemptner and others have found appreciable amounts of copper in the blood following injections of copper salts. They

have reported that no trace of copper could be found in the serum but all in the blood cells. Chemical examinations of the blood of eight pigs which had been injected subcutaneously gave results as shown in table 4. The blood was drawn from the heart six hours after the injection.

In two of the pigs, one injected with copper sulphate, the other with copper glutaminate, there was an hemorrhagic peritoneal exudate which on analysis showed a copper content of 0.01 mgm. for the former and 0.059 mgm. for the latter. The autopsy findings in all these pigs are the same as given above.

Chemical analyses for copper were made on the viscera of some pigs which were injected subcutaneously with the lethal dose of the four copper salts. The urine and feces were collected for a period of four days before injection, and the urine

TABLE 4

Blood analyses for copper after subcutaneous injection

	COPPER SALT							
	$CuSO_4$		Cu g'ucinate		Cu glutaminate		Cu leucinate	
Milligram of Cu injected per kilo	4.0	8.0	4.0	8.0	4.0	8.0	4.0	8.0
Blood analyzed (cubic centimeter)	6.0	16.0	10.0	10.0	8.0	5.0	8.0	5.0
Copper found	0.0	trace	0.0	0.0	0.0	0.0	0.0	0.0

after the injection, and copper analyses made on these collections. The results of these analyses are given in table 5.

The autopsy findings in all these pigs are similar to those given above. There were no feces in any case after the injection and in pigs 3, 6, 7, 9 and 10, the urine after the injection appeared bloody. As so much copper was found in the feces and in the gastro-intestinal tract, analyses of specimens of the oats and carrots which were fed to the pigs were made and appreciable amounts of copper were found in both, 4.2 mgm. of copper per kilo in oats and 1.92 mgm. per kilo in carrots. These figures are somewhat lower than those reported by other investigators, but many have shown that regional differences exist.

Taken as a whole, this series of experiments indicates that

TABLE 5

Copper in the viscera after subcutaneous injections

	COPPER SALT									
	Control		Cu-leucinate		Cu-glutaminate		Cu-glycinate		Cu-sulphate	
Pig number	1	2	3	4	5	6	7	8	9	10
Milligram of Cu injected per kilo			12	10	12	10	12	10	12	10
Death after injection			4 hours	4½ hours	7 hours	8 hours	5 hours	1 hour	7 hours	14 hours
Total milligram of copper in feces before			0.326	0.356	0.174	0.122	0.732	0.136	0.538	0.033
Total milligram of copper in urine before			0.006	0.000	0.000	0.000	0.001	0.000	0.022	0.00
Total milligram of copper in urine after			0.035	0.006	0.000	0.000	0.396	0.000	0.302	0.198
Total milligram of copper in stomach and contents			0.196	0.137	0.148	0.162	0.191	0.174	0.149	0.078
Total milligram of copper in large intestines and contents			0.296	0.382	0.332	0.221	0.198	0.410	0.628	0.356
Total milligram of copper in small intestines and contents			0.280	0.252	0.245	0.292		0.502		0.220
Milligram of copper per kilo in:										
Lung	0.000	0.000	0.020	0.05	0.001	0.02	0.003	0.001	0.06	0.000
Spleen	0.000	0.000	0.000	0.000	0.000	0.000	0.01	0.000	0.49	0.000
Heart	0.000	0.000	0.001	0.032	0.000	0.000	0.018	0.000	0.07	0.000
Kidney	0.010	0.000	0.020	0.510	0.240	0.640	0.410	0.210	0.49	0.420
Liver	0.045	0.035	0.136	0.160	0.135	0.180		0.180	0.17	0.380

there are no differences in the action of the copper salts used, although the leucinate in a few cases seemed more toxic. The lower copper value in the feces of pigs 6, 8, and 10, is probably due to a change in the diet to new carrots, during the experiment. The finding of copper in the urine of pigs 3, 4, 7, 9, and 10, after the injection shows that the kidneys excrete small quantities of this metal. This observation has been made by others, while Chittenden never found copper in the urine of experimental animals that were fed copper preparations although the kidneys showed an appreciable amount of copper. The urine of pigs 3, 7, and 9 showed traces of copper before the injection and much larger amounts after the injection. This may mean that the pigs were utilizing a part of the copper in the normal diet. Pigs 4 and 8 did not excrete urine after the injection. The finding of large amounts of copper in the alimentary tract and its contents is of uncertain value, as the foods which were used contained some copper. The analyses of the other viscera, however, although not showing constant variations for the different salts used, show that the liver and the kidney are the chief organs in which copper is found; in all cases but one the kidney containing a higher percentage of copper than the liver. The blood analyses show that the injected copper disappears very rapidly from the blood stream. Klemptner has found in experiments *in vitro* that the copper is attached to the red blood cells and is not in the serum. If this is true, the red blood cells to which the copper is attached must be removed from the blood stream within a short time.

DIALYSIS OF COPPER SALTS THROUGH CELLOIDIN SACS

Kober and Sugiura have noted that the copper salts of the amino acids are dialyzable and Filehne has reported that there are marked differences in the rate of dialysis of certain copper compounds. Many observers have reported that copper easily forms complex protein molecules when brought in contact with proteins, and experimentation has shown that when vegetables are greened with copper two complex copper compounds are

formed. The question naturally arises as to the fate of copper introduced directly into the blood stream or taken up after injection or from the gastro-intestinal tract. Does the copper form a complex protein compound and, if so, is this compound able to penetrate cell membranes? In order to throw more light on the action of certain copper compounds a series of

TABLE 6

Dialysis of copper salts with serum

	COPPER SALT							
	Cu-leucinate		Cu-glutaminate		Cu-glycinate		Cu-sulphate	
Milligram of Cu used.....	0.402	0.403	0.472	0.476	0.468	0.460	0.516	0.506
Horse serum inside (cubic centimeters)	10	10	10	10	10	10	10	10
Horse serum outside (cubic centimeters).....	33	33	33	33	33	33	33	33
Per cent of Cu dialyzed....	59.8	58	60	58.7	57.8	5.9	58.4	59.7

TABLE 7

Dialysis of copper salts with distilled water

	COPPER SALT							
	Cu-leucinate		Cu-glutaminate		Cu-glycinate		Cu-sulphate	
Milligram of Cu used.....	0.402		0.472		0.464		0.508	
H_2O inside (cubic centimeters.....	10	10	10	10	10	10	10	10
H_2O outside (cubic centimeters	33	33	33	33	33	33	33	33
Per cent of Cu dialyzed....	71.5	70.4	67	68	70.8	71	68	70

dialysis experiments was conducted. The copper salts were made up in the same molar concentration and dialyzed in serum and in distilled water solutions. In one series horse serum was used on both sides of the celloidin sac and in another series distilled water was used on both sides. In both series the copper was added to the serum and to the distilled water and incubated at body temperature for six hours, then was transferred to the dialyzing sac and incubated for twelve hours at body temperature. Analyses were then made to determine the amount of copper in the fluid outside the dialyzing sac. The results of the dialyses are given in tables 6 and 7.

Slight variations with the same salt are probably due to slight differences in the thickness of the dialyzing sacs. The figures show that there is no appreciable difference in the rate of dialysis with the four salts tested and very little difference between the distilled water and horse serum dialyses. These findings then, oppose the view that these copper salts, when brought in contact with protein materials, form complex protein molecules which are not readily dialyzable. This fact may explain the rapid disappearance of copper from the blood stream as noted above and is also in accord with the finding of copper in peritoneal and pleural exudates after injections of copper.

EXPERIMENTAL CHRONIC COPPER INTOXICATION

The production of a typical chronic copper intoxication has been disputed for many years and even today there are many who state that a chronic copper intoxication cannot be produced. There seem to be good grounds for contention, as those who oppose the view that copper may produce a chronic intoxication point to the numerous examples of workers in copper who show no symptoms of intoxication, even after they are so saturated with copper that their hair, nails, gums, and bones are colored greenish. They contend that the apparent chronic copper intoxication is caused by some other agent and is not due to the copper. On the other hand, numerous experimenters with animals have shown that, in animals, at least, certain definite pathological changes follow long continued ingestion of copper and that these changes are accompanied by the deposition of copper in certain tissues. Taylor has recently shown that small amounts of copper may be ingested for a long period by man without producing any distinguishable nutritional disturbances. However, with reference to the retention of copper in the liver, he says, "I do not believe such a retention of heavy metal can be a negligible matter even in the complete absence of present symptoms referable thereto." As such different conclusions have been drawn by different observers, it is almost impossible to interpret the final results. This discrepancy in findings may

be partially explained by the different methods used by these investigators. Many results can not be interpreted as the observers have failed to state all of the conditions of the experiment. It is necessary to know the dosage per kilo body-weight, the method of ingestion, the species of animal, and the duration of the experiment before any conclusions can be accepted. Table 8 summarizes briefly some of the work bearing on the question of chronic copper intoxication.

This table shows the marked variations in the conditions of the experiments which may explain the different results obtained. Small dosages are recommended by Baum and Seeliger, Lewin, Meyerhardt, Chittenden and others, as it was found that larger amounts of copper were absorbed with small doses than with larger doses and it was assumed that the larger doses produced a chronic catarrhal inflammation of the gastro-intestinal tract which interfered with absorption. The question of the variation in toxicity of the different copper compounds accounts for the difference in dosage used by different workers. Lehmann, Mock, Tschirch, Demme and Lang, and Taylor could find no difference in the toxicity of the different copper compounds that they used, while Feltz and Ritter, Filehne, Brandl and Baum, and Seeliger found marked variation in the toxicity of different salts, depending on the copper preparation used. Because of the different views as to the toxicity of the different copper compounds, it seemed advisable to test the toxicity of the four salts used in this present experimental work by the chronic intoxication method. The experimental part of the work is divided into three parts, the attempt to produce chronic intoxication by (1) subcutaneous injections, (2) intramuscular injections and (3) feeding.

SUBCUTANEOUS INJECTIONS

As I have found no records of attempts to produce a chronic copper intoxication by making subcutaneous injections, the dosage used was calculated on the relative values of subcutaneous injection and feeding doses in the production of acute copper intoxication. Filehne found that this ratio was about

TABLE 8

Chronic copper intoxication by feeding

OBSERVER	DATE	Cu PREPARATION	MILLIGRAMS OF Cu PER KILO PER DAY	LENGTH OF EXPERIMENT	ANIMAL USED	RESULTS
				days		
Ellenberger and Hofmeister	1883	$CuSO_4$	10 0	140	Sheep	Pathological changes in liver and kidney
Ellenberger	1898	$CuSO_4$			Dogs	Pathological changes in liver and kidney
		Cu haemol			Sheep	
		Cu acetate			Cats	
		Cu oleate			Goats	
Filehne	1895	CuNa tartrate	11.0	16	Rabbit	Pathological changes in liver and kidney
	1896	Cu stearate	4.0	60	Dogs	
		Cu metallic	4.0	60	Dogs	
Brandl	1893	CuNa tartrate		15	Rabbit	Pathological changes in liver and kidney
		CuO		37	Rabbit	
		Cu haemol	6.9		Dog	
		Cu oleate	7.7	14	Dog	
		$CuSO_4$		130	Rabbit	
Baum and Seeliger	1898	Cu haemol	7.7–12 5	220	Dogs	Pathological changes in small intestine, liver and kidney
		$CuSO_4$	2 0– 3.5	220	Dogs	
		Cu acetate	0 8– 5 0	220	Dogs	
		Cu oleate	2 5– 3.0	220	Dogs	
Trolldeimer	1897	Cu haemol	3.5	220	Dog	Pathological changes in liver and kidney
		Cu acetate	12.0	28	Cat	
		$CuSO_4$	3.0	220	Dog	
		Cu oleate		13	Cat	
Chittenden	1913	Coppered peas	0.30– 0 4	60	Dogs	Pathological changes not noticeable
		$CuSO_4$	0.35	60	Dogs	

one to fifteen. Others have found the ratio to be about one to twelve. If the average dose per os to produce a chronic intoxication is taken at 4.5 mgm. of copper per kilo per day, the subcutaneous injection will be about 0.30 mgm. copper per kilo per day. This dose, however, is as large as that given per os by Chittenden but he had a special reason for giving such a small dose. In this series of experiments, injections were made into the loose tissue of the axillae and the groins, being so arranged that every fifth injection was made in the same area. At first the injections were made at four day intervals and later at three day intervals, so that about twenty days elapsed between injections at any one place. The injections were made with a 1 cc. tuberculin syringe, as olive oil emulsions

TABLE 9

Subcutaneous injections of copper

	CU SALT							
	Copper Leucinate		Copper glutaminate		Copper sulphate		Controls	
Pig number	11	12	13	14	15	16	17	8
First weight (grams)	390	400	390	410	280	500	400	310
Weight at death (grams)	620	670	620	700	625	715	640	500
Milligrams of Cu injected	16.7	16.7	16.7	16.7	16.7	16.7	0	0

of the copper salts containing 2.0 mgm. of copper per cc., from May 18 to August 30, the pigs having received in that time about 17 mgm. of copper.

All the pigs at the time of death, one hundred and two days after the beginning of the experiment, were sleek and fat and at no time had there been any ulceration at the points of injection. Three days after the last injection the pigs were killed and tissues from the liver, spleen, kidney, heart and lung preserved in Zenker's fluid for histological examination and the remainder of these organs taken for chemical analysis. The results of the chemical analyses are given in table 10. The autopsy findings showed no gross pathological changes, except that in three pigs the kidneys showed a few small cysts.

TABLE 10

Copper content of tissues after subcutaneous injections

	COPPER SALT							
	Leucinate		Glutaminate		Sulphate		Control	
Pig number	I	II	I	II	I	I	I	II
Milligram of Cu. per kilo in								
Spleen	0.08	0.03	0.05	0.000	0.000	0.000	0.000	0.006
Heart	0.06	0.05	0.13	0.16	0.20	0.12	0.000	0.000
Lung	0.03	0.04	0.04	0.01	0.000	0.008	0.000	0.000
Kidney	0.23	0.24	0.22	0.19	0.09	0.19	0.04	0.000
Liver	1.35	0.69	0.58	0.49	0.55	0.31	0.039	0.036

INTRAMUSCULAR INJECTIONS

The same scheme of injection was used in these experiments as was used in the subcutaneous injections, the only difference being that the inoculations were made into the muscles of the fore and hind legs. The duration of the experiment and the total amount of copper injected were the same as in the subcutaneous experiment and similar examinations and analyses were made. The autopsy findings showed nothing abnormal. The results of the chemical analyses are given in table 11.

TABLE 11

Copper content of tissues after intramuscular injection

	COPPER SALT							
	Leucinate		Glutaminate		Sulphate		Control	
Pig number	I	II	I	II	I	II	I	II
Milligram of Cu per kilo in:								
Spleen	0.15	0.14	0.06	0.13	0.000	0.14	0.000	0.006
Heart	0.03	0.000	0.000	0.000	0.000	0.07	0.000	0.000
Lung	0.01	0.07	0.10	0.10	0.000	0.04	0.000	0.000
Kidney	0.12	0.15	0.06	0.21	0.09	0.11	0.04	0.000
Liver	0.49	0.55	0.69	0.57	0.54	0.87	0.03	0.036

The histological examination of the tissues, liver, kidney, spleen, myocardium and lung, did not show any constant changes when compared with the tissues of control animals. Trolldenier, Ellenberger and Hofmeister, Brandl, Klemptner and De Moor found certain changes, especially in the liver and kidney but

the changes noted by them varied considerably. Some, Smith, Trolldenier, even noticed that the changes were more marked in some salts than in others but in our tissues no differences could be made out either for the different salts or for the different modes of injection. Certain inconstant changes were noted; the Malpighian bodies of the spleen were very prominent in some sections, the lungs showed frequently markedly thickened alveolar walls and many small collections of round cells, the kidneys often contained small foci of round cells and a slight increase in connective tissue in the cortex, but the hemorrhages and brownish granules spoken of by Klemptner, Ellenberger and Hofmeister were absent, the livers seemed less fatty in the fed than in the control animals and the collections of brownish pigments and evidences of periportal inflammation spoken of by many were not observed. Taken as a whole, the findings in these tissues in general, corroborate the findings of Smith, i.e., that the tissue changes are not characteristic, although analyses show that the copper is deposited in the tissues.

These analyses show, as Corper has previously noted, that copper, which is introduced subcutaneously or intramuscularly, is deposited chiefly in the liver and to a much less degree in the kidney.

This deposition in the different tissues is not influenced by difference in the form of copper or by the different methods of introduction and, as shown in the histological report, causes no appreciable changes in the tissue.

FEEDING

The summary of chronic copper intoxication given above shows the marked variation in the dosage of copper per day per kilo used by the different workers where the main purpose of the experiment was to produce a chronic copper intoxication. As the main purpose of the present experiment is to determine whether the different copper salts used show different degrees of toxicity, an average sized dose, based on the work of others, was used, the dose being the same for all the salts. These salts

were fed to guinea pigs in pill form, each pill containing two mgm. of copper with a vehicle of graham crackers, olive oil, flour and water. These pills were fed at first daily, in the morning; then twice daily, morning and afternoon; then three times daily, morning, noon and afternoon. This method of feeding insured at least one feeding per day on an empty stomach, at which time absorption is more complete. Controls were given the same ration, minus the copper. These pigs were fed about 360 mgm. of copper in seventy-two days. At the close of the experimental period the animals were killed by bleeding, examined for gross pathological changes, tissues preserved in Zenker's fluid for histological examination, the other tissues kept for chemical analysis. The following table shows the results of these examinations.

The results of the attempts to produce a chronic intoxication by feeding the copper salts named above, are similar to those obtained by the injection methods, the distribution and amount of copper being practically the same regardless of the method used. The histological picture of the tissues where copper is deposited also fails to show any changes which can be attributed to the copper. Fatty changes in the liver and kidney were very inconstant and showed no relation to the amount of copper deposited. Six of the twelve pigs died from various causes (intestinal intoxication, puerperal infection, advanced tuberculosis) or were killed before the completion of the feeding period and complete examinations of these were made at the time of death. Three of these and three of those killed at the end of the feeding-period had experimental tuberculous lesion in the lungs and two had tuberculous lesion in the liver and spleen. Analyses of these tuberculous organs failed to show a higher concentration of copper than was found in the non-tuberculous pigs. One large caseous nodule in the liver contained only about one-fifth as much copper as the surrounding liver tissue. These findings are not in accord with those of the German investigators who used certain copper combinations in the treatment of skin and pulmonary tuberculosis, but corroborate the findings of Corper.

TABLE 12

Copper content of tissues after feeding

	COPPER SALT													
	Leucinate			Glutaminate			Glycinate			Copper sulphate			Controls	
Pig number	I	II	III	I	II	III	I	II	III	I	II	III	I	II
Time fed, days	18	72	65	44	72	72	72	58	72	51	15	72	72	72
Milligram of copper per kilo in:														
Spleen	0.00	0.00	0.00	0 00	0.00	0.00	0.00	0 000	0.000	0 00	0.00	0.000	0.000	0.00
Heart	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0 000	0.000	0.00	0 00	0.002	0.000	0.00
Lung	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.005	0.000	0.00	0.00	0.000	0.000	0.00
Kidney	0.00	0.00	0.00	0.03	0.14	0 04	0.15	0.010	0.015	0.01	0.00	0.040	0.001	0.00
Liver	0.59	0.31	0.87	0.56	0.34		0.40	0.700	0.500	0.36	0.26	0.680	0.040	0.03
Blood		0.00			0.02	0.00	0.00		0.010			0.000	0.000	0.00
Changes in weight, in grams	Gain	Lost	Lost	Lost	Gain	Gain	Gain	Lost	Lost	Lost	Gain	Lost	Lost	0 00
	50	25	240	10	25	160	105	60	20	150	15	60	150	

It is of interest to note that the livers of pigs newly born of mothers on the copper diet contained almost the same proportion of copper that the mothers' liver contained, while the remainder of the body contained only a trace of copper. This shows definitely, as has been pointed out by others, that copper passes through the placenta.

GENERAL SUMMARY

1. Copper, whether in the form of the sulphate, leucinate, glycinate or glutaminate, shows no variations in action when introduced into the conjunctiva of rabbits. The lower dilutions, 1 per cent, produce hyperemia and lacrimation to the same degree, while the higher dilutions are inactive.

2. Copper, in the salt forms named above, shows very little if any variation in ability to produce acute intoxication when introduced in dilute solutions, subcutaneously. The dilution is important as the local injury produced by the higher concentrations interferes with absorption. The toxic subcutaneous dose of all the salts was found to be between 4 to 6 mgm. per kilo for guinea pigs.

3. Copper, in the salt forms named above, shows no variations in action when introduced intracutaneously. The lower dilutions cause necrosis and induration and the higher dilutions cause little or no change. The histological picture with the same dilution of all salts is the same.

4. Copper, in the salt forms named above, shows no variation in action when introduced in small gradually increasing doses, 0.5 to 1 mgm. copper per kilo, for a long period of time, one hundred and five days, either subcutaneously or intramuscularly. This dosage of copper seems to produce no gross or microscopic changes in the liver, kidney, spleen, heart, or lungs, although the liver and kidney both show marked increase in amounts of copper when analyzed.

5. Copper, in the salt forms named above, shows no variation in ability to dialyze through celloidin sacs when either water or horse-serum are used both inside and outside the sac.

The rate of dialysis is somewhat faster with distilled water than with horse serum.

6. Copper, in the salt forms named above, shows no variation in action when introduced by feeding in small gradually increasing doses, to 10 mgm. per kilo per day, for a long time, seventy-two days. The deposition of copper in the liver and kidney and the lack of gross and microscopic changes in the tissues examined, simulate the findings in those experiments where the copper was introduced subcutaneously or intramuscularly.

7. This experimental work seems to show that the three copper amino acids examined produce exactly the same physiological effects as a simple inorganic salt, copper sulphate.

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